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Integrin-mediated Interactions Between Human Bone Marrow Stromal Precursor Cells and the Extracellular Matrix

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To date, the precise interactions between bone marrow stromal cells and the extracellular matrix that govern stromal cell development remain unclear. The integrin super-family of cell-surface adhesion molecules represents a major pathway used by virtually all cell types to interact with different extracellular matrix components. In this study, purified populations of stromal precursor cells were isolated from the STRO-1-positive fraction of normal human marrow, by fluoresence-activated cell sorting, and then assayed for their ability to initiate clonogenic growth in the presence of various integrin ligands. Bone marrow-derived stromal progenitors displayed differential growth to fibronectin, vitronectin, and laminin, over collagen types I and III, but showed a similar affinity for collagen type IV. The integrin heterodimers $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ were found to coexpress with the STRO-1 antigen on the cell surface of CFU-F, using dual-color analysis. Furthermore, only a proportion of stromal precursors expressed the integrin $\alpha_4\beta_1$, while no measurable levels of the integrin $\alpha_3\beta_1$ could be detected. Subsequent adhesion studies using functional blocking antibodies to different integrin α/β heterodimers showed that stromal cell growth on collagen, laminin, and fibronectin was mediated by multiple β_1 integrins. In contrast, cloning efficiency in the presence of vitronectin was mediated in part by $\alpha v\beta 3$. When human marrow stromal cells were cultured under osteoinductive conditions, their ability to form a mineralized matrix in vitro was significantly diminished in the presence of a functional blocking monoclonal antibody to the $\beta 1$ integrin subunit. The results of this study indicate that $\beta 1$ integrins appear to be the predominant adhesion receptor subfamily utilized by stromal precursor cells to adhere and proliferate utilizing matrix glycoproteins commonly found in the bone marrow microenvironment and bone surfaces. Furthermore, these data suggest a possible role for the $\beta 1$ integrin subfamily during the development of stromal precursor cells into functional osteoblast-like cells. (Bone 28: 174–181; 2001)

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Introduction

The nonhematopoietic connective tissue of the bone marrow (BM) microenvironment is derived from a heterogeneous population of stromal precursor cells, whose progeny support and regulate haematopoiesis.[2,4,40,60,62] A proportion of this precursor population demonstrate "stem cell-like" characteristics, with the capacity to differentiate into many of the stromal cell lineages found within the bone marrow spaces and the adjacent calcified tissues of bone and cartilage.[20,22,43,45] The molecular mechanisms that govern stromal cell commitment and differentiation are poorly understood and are only now starting to be elucidated.[14,17,37,41] Stromal cell development and function is, in part, mediated by a complex series of cell-cell and cell-extracellular matrix (ECM) interactions, acting through a myriad of ECM protein ligands and their corresponding cell surface receptors, including membrane-bound cytokines.[8,9,12,18,57,64]

Integrins represent a major family of cell-surface receptors that facilitate the adhesion between cells and the surrounding ECM. Interactions between integrins and their ligands have been linked to many cellular processes including, proliferation, differentiation, survival, motility, embryogenesis and apoptosis.[5,6,13,34,52,54] Integrin molecules are transmembrane heterodimers comprised of noncovalently bound α- and β-subunits. There are at least 16 different α subunits associated with as many as nine known β subunits.[6,9,32,34,47,52,54] Many α subunits, such as $\alpha 1$, are found in association with only one β subunit ($\alpha 1\beta 1$), whereas others, such as αv, demonstrate promiscuity by associating with multiple β subunits ($\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$) to generate a different ligand binding specificity for each heterodimer pair. There is also a high level of redundancy with respect to ligand specificity between the different integrin species, and even within the same integrin subclass. These properties endow integrins with the potential to elicit a large number of different cellular responses, depending on the type of integrin receptor expressed, the developmental stage of the cell, and according to the composition of the surrounding extracellular matrix.[5,9,36,47,54] The complexity of cell-cell and cell-matrix interactions presents a major challenge to unraveling the exact mechanisms that facilitate stromal cell function and development, where virtually nothing is known of the interactions

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between stromal precursor cells and the extracellular matrix in vivo.

Early studies first identified BM stromal precursor cells by their ability to form clonogenic cell clusters comprised of fibroblast-like cells (colony-forming unit fibroblast [CFU-F]) in vitro.[7,19,21,44,46] We have subsequently shown that human BM stromal cells, with clonogenic potential in vitro, react with the monoclonal antibody STRO-1, using fluorescence-activated cell sorting (FACS).[23,56] Stromal cultures derived from STRO-1-positive sorted BM cells have the capacity to support long-term hematopoiesis and the potential to develop into different mature stromal cell types including fibroblasts, smooth muscle cells, adipocytes, and osteoblasts.[24,56] The present study examines the mechanisms mediating the growth of purified CFU-F on different ECM components, typically found in the loose meshwork of marrow reticular fibers (collagen type III), the endothelial basal laminae of sinusoids (collagen type IV and laminin), and the surrounding, calcified matrix of bone (collagen type I). A systematic immunophenotypic analysis was compiled to identify the pattern of integrin expression on the cell surface membrane of CFU-F, isolated from aspirates of normal human BM. The functional significance of receptors expressed by CFU-F was then examined, using a panel of function-blocking monoclonal antibodies against a range of integrin heterodimers. The importance of integrin function in stromal cell development was also addressed in the presence of a β1 integrin-blocking antibody, under osteoinductive conditions in vitro.

Materials and Methods

Subjects

Aspirates of human BM samples were obtained from the iliac crest and the sternum of normal adult volunteers with their informed consent, according to procedures approved by the Human Ethics Committee at the Royal Adelaide Hospital, South Australia and the NIH/NIDCR Human Ethics Committee (protocol no. 94-D-0188). Bone marrow mononuclear cells (BMMNC) were obtained by centrifugation over Ficoll 1.077 g/mL (Lymphoprep, Nycomed, Oslo, Norway) at 400 *g* for 30 min and then washed and resuspended with Hank's buffered saline solution (HBSS) containing 1% bovine serum albumin (BSA) (Cohn Fraction V; Sigma Chemical Co., St. Louis, MO) and 10 mmol/L Hepes.

Colony-forming Efficiency (CFU-F) Assay

Details of these assays have been described elsewhere.[36,38] For serum-replete cultures, STRO-1^+ BMMNC were isolated by FACS, then plated in alpha modification of Eagle's medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 20% fetal calf serum (Equitech-Biol Inc., Kerrville TX), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate (Sigma), and β-mercaptoethanol (5×10^{-5} mol/L). In serum-free cultures of STRO-1^+, BMMNC isolated by FACS were plated in α-MEM supplemented with 10 ng/mL PDGF-BB and EGF (Pepro Tech Inc., Rocky Hill, NJ), 10 μg/mL bovine pancreas-derived insulin (Sigma), 2% BSA, 4 μg/mL human low-density lipoprotein (LDL; Sigma, L121139), 200 μg/mL iron-saturated human transferrin, 2 mmol/L L-glutamine, dexamethasone sodium phosphate (10^{-8} mol/L) (DEX; American Regent Laboratories, Inc., Shirley, NY), 100 μmol/L L-ascorbic acid 2-phosphate (ASC-2P; Novachem, Melbourne, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma), and β-mercaptoethanol (5×10^{-5} mol/L). Cell attachment under serum-free conditions was promoted by precoating the tissue culture plates with purified matrix proteins as described below. Cultures were established as triplicates for each condition and incubated at 37°C in 5% CO_2 in air for 14 days. On day 14 of culture, the cells were washed twice with warm RPMI-1640 and then fixed for 20 min in 1% phosphate-buffered formalin prepared from paraformaldehyde (PBF). Once fixed, the cultures were stained with 0.1% toluidine blue (in 1% PBF) for 1 h, then rinsed in tap water. Aggregates of 50 cells or more were scored as colonies (progeny of individual CFU-F).

Monoclonal Antibodies

HB245 (anti-$\alpha_1\beta_1$ $mIgG_1$ 1/4) and AC11 (anti-$\alpha_2\beta_1$ $_mIgG_1$ 1/50) were obtained from the American Type Culture Collection (Rockville, MD) P1B5 (anti-$\alpha_3\beta_1$ $mIgG_1$ 1/4) was a generous gift from Dr. E. Wayner University of Minnesota Medical School (St. Paul, MN). P4G9 (anti-$\alpha_4\beta_1$ $mIgG_1$ 1/4) was from Telios Pharmaceuticals Inc. (San Diego CA). PHM2 (anti-$\alpha_5\beta_1$ $_mIgG_1$ 1/500) was a generous gift from Prof. R. Atkins, Department of Human Immunology (H.C.C.R., I.M.V.S., Adelaide, South Australia). 61.2C4 (anti-β_1 $mIgG_1$ 1/4) was a generous gift from Dr. J. Gamble Department of Human Immunology (H.C.C.R., I.M.V.S., Adelaide, South Australia). GoH3 (anti-$\alpha_6\beta_1$ $rIgG_{2a}$ 1/4), MCA1212 (negative control antibody $rIgG_{2a}$ 1/10), 2C36 (anti-$\alpha_v\beta_3$ $mIgG_1$ 1/4), and PH59 (anti-$\alpha_v\beta_5$ $mIgG_1$ 1/4 nonfunctional) were obtained from Serotec (Oxford, England). Y2/51 (anti-β3 $mIgG_1$ 1/4 nonfunctional) was from Dakopatts A/S (Glostrup, Denmark). 3D3 (anti-*Salmonella* $mIgG_1$ 1/4), 1A6.12 (anti-*Salmonella* mIgM 1/4), and 1D4.5 (anti-*Salmonella* $mIgG_{2a}$ 1/4) were the generous gifts from Dr. L. Ashmann, Department of Haematology (H.C.C.R., I.M.V.S., Adelaide, South Australia). STRO-1 (anti–marrow stromal progenitor cells mIgM 1/4) was from Dr. P. J. Simmons, Department of Haematology (H.C.C.R., I.M.V.S., Adelaide, South Australia).

Fluorescence-activated Cell Sorting (FACS)

After Ficoll gradient seperation, approximately 2×10^7 BMMNC were pelleted in 5-mL polypropylene tubes (Falcon; Becton Dickinson, Linkon Park, NJ) and resuspended in 500 μL of saturating concentrations of STRO-1 supernatant for 60 min on ice. For two-color FACS, 2×10^7 cells were incubated with STRO-1 supernatant and various mouse IgG anti–human monoclonal antibodies. The monoclonal mouse isotype controls IgG_1 (3D3), IgG_{2a} (1D4.5), and IgM (1A6.12) and rat isotype control IgG_{2a} (MA1212) were under identical conditions. The cells were then washed in HBSS + 5% FCS and incubated with a goat anti–mouse IgM μ-chain-specific fluorescein isothiocyanate (FITC) (1/30) (Southern Biotechnology Associates, Birmingham, AL) in a final volume of 100 μL for 45 min on ice. For two-color FACS analysis, the cells were also incubated with a goat anti–mouse IgG γ-chain-specific phycoerythrin (PE) (1/50) (Southern Biotechnology Associates, Birmingham, AL). After this, the cells were washed twice in HBSS + 5% FCS and resuspended to approximately 10^6 cells/mL before being analyzed using a FACStar^{PLUS} (Becton Dickinson, Sunnyvale, CA) flow cytometer. Positive fluorescence for each antibody was defined as the level of fluorescence greater than 99% of the corresponding isotype-matched control antibody.

Analysis of CFU-F Growth on Purified ECM Proteins

Non–tissue culture grade 48-well polystyrene plates (Nunc) were precoated with various purified ECM proteins: collagen type I (167 nmol/L), collagen type III (167 nmol/L), collagen type IV (45 nmol/L) (Collaborative Research Inc., Two Oak Park, Bed-

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ford, MA), laminin (56 nmol/L), fibronectin (114 nmol/L) (Boehringer Mannheim GmbH, Germany), and 1% BSA (Cohn fraction V; Sigma) and incubated overnight at 4°C. The plates were then rinsed three times with PBS and blocked at 37°C for 2 h with 1% BSA in PBS. Bone marrow aspirates were separated by density gradient and then washed in HBSS containing 1% BSA. STRO-1^+ BMMNC were isolated by FACS and subsequently seeded ($2 \times 10^4/cm^2$) as triplicate cultures in 250 μL of serum-free media (SFM) containing 10 ng/mL of PDGF-BB and EGF at 37°C in 5% CO_2 for 2 h. After this, each well was washed three times (with the exception of the positive control wells) with SFM to remove any unbound cells and then replenished with 500 μL of SFM + PDGF-BB and EGF and incubated at 37°C in 5% CO_2 for 14 days. Cultures were then fixed and stained for 60 min with 0.1% Toluidine blue in 1% PBF. Aggregates of ≥50 cells were scored as CFU-F colonies. Preliminary time course experiments demonstrated that only a minor fraction of the total number of colony-forming cells were recovered within the first half hour (10–25%) and hour (30–50%) of incubation under serum-free conditions for all proteins tested. Maximal recovery (>90%) of CFU-F for each matrix protein was observed between 4 and 6 h, in contrast to the 90-min incubation time required in the presence of serum.[7] Herein, all adhesion assays were performed at 2 h, which consistently produced 60–70% recovery of CFU-F with clonogenic potential.

Measurement of Calcium Levels

We have previously reported the conditions for the induction of human bone marrow stromal cells to initiate matrix mineralization in vitro.[24] Briefly, primary BM stromal cells were seeded onto 96-well microtitre plates (15×10^3 cells/cm^2), in α-MEM supplemented with 20% FCS, L-glutamine (2 mmol/L), β-mercaptoethanol (5×10^{-5} M), L-ascorbic acid 2-phosphate (100 μmol/L) (Novachem, Melbourne, Australia), dexamethasone sodium phosphate (10^{-8} mol/L) (American Regent Laboratories, Inc., Shirley, NY), KH_2PO_4 (1.8 mmol/L) (BDH Chemicals), and Hepes (10 mmol/L), at 37°C, 5% CO_2. The media were supplemented with either the negative control monoclonal antibody 3D3 (100 μg/mL) or the anti-β1 monoclonal antibody (61.2C4 at 100 μg/mL). Cultures were fed twice a week with fresh medium and antibody for a period of 4-weeks. Calcium levels were determined as previously described at weekly intervals. After this, the cultures were washed four times with Ca^{2+} and Mg^{2+} free PBS and then solublized with 0.6 N HCl (100 μL per well). Samples from each well were then reacted with *o*-cresol-phthalein-complexon (Test Combination Calcium; Boehringer Mannheim), and the colormetric reaction was read at 570 nm. The absolute calcium concentration was determined according to a standard curve for calcium according to the manufacturer's recommendations.

Statistical Analysis

The paired *t*-test was used to determine any significant differences ($p \leq 0.05$) between the clonogenic growth of BM CFU-F cultured on different matrix proteins. Similarly, colony growth was compared for each matrix protein coating between cells pretreated with various combinations of functional-blocking integrin monoclonal antibodies in comparison with the corresponding isotype-matched control monoclonal antibodies. Statistical significance ($p \leq 0.05$) in the level of calcium detected between the anti-β1-treated BM stromal cell cultures and the corresponding isotype control antibody was also determined using the paired *t*-test.

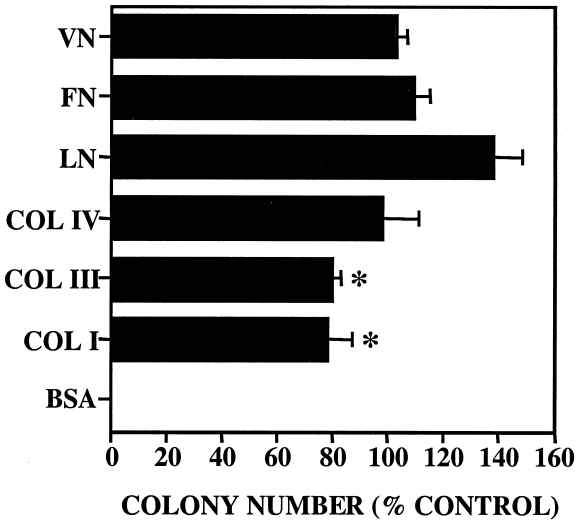

Figure 1. Human BM CFU-F interactions with different extracellular matrix glycoproteins. STRO-1-positive BM cells were isolated by FACS and then plated onto 48-well non-tissue culture-treated plates precoated with purified collagen types I (COL I), III (COL III), and IV (COL IV), fibronectin (FN), laminin (LN), and vitronectin (VN) under serum-deprived conditions as described in Materials and Methods. Clonogenic frequencies were determined for all conditions. The data represent the mean values (± SEM) from three experiments expressed as a percentage of the number of colonies formed by the input population in the control cultures grown in the presence of 20% fetal calf serum. Significant differences (*$p \geq 0.05$, *t*-test) in colony numbers were assessed between the different conditions.

Results

Growth of Stromal Precursor Cells on Purified ECM Glycoproteins

To investigate the interactions between marrow stromal precursor cells and extracellular matrix components, freshly sorted STRO-1^+ BM cells were seeded onto different purified matrix proteins and assessed for their potential to form colonies under serum-free conditions in the presence of PDGF and EGF. Cloning efficiencies under serum-free conditions were found to be comparable with the control cultures grown in the presence of 20% serum (**Figure 1**). There was no difference in the cloning efficiency of the BM CFU-F cultured on collagen type IV, fibronectin, laminin, and vitronectin. However, CFU-F demonstrated a significant reduction ($p \leq 0.05$, paired *t*-test) in the number of colonies formed when seeded on collagen types I and III, in comparison with fibronectin, laminin, and vitronectin. In addition, no obvious variations in cell morphology were observed between colonies generated from CFU-F plated on the different matrix proteins, with the exception that colonies appeared larger on those plates coated with laminin (data not shown).

Integrin Expression on BM Stromal Precursor Cells

Dual-color FACS analysis was employed to subdivide the STRO-1^+ BMMNC fraction based on reactivity to a range of monoclonal antibodies specific to different integrin αβ heterodimers (**Figure 2**). Clonogenic growth of BM stromal cells was measured in the different sorted STRO-1/integrin subfractions and was expressed as a percentage of the total number of colonies in the high-expressing STRO-1 population (**Figure 3**),

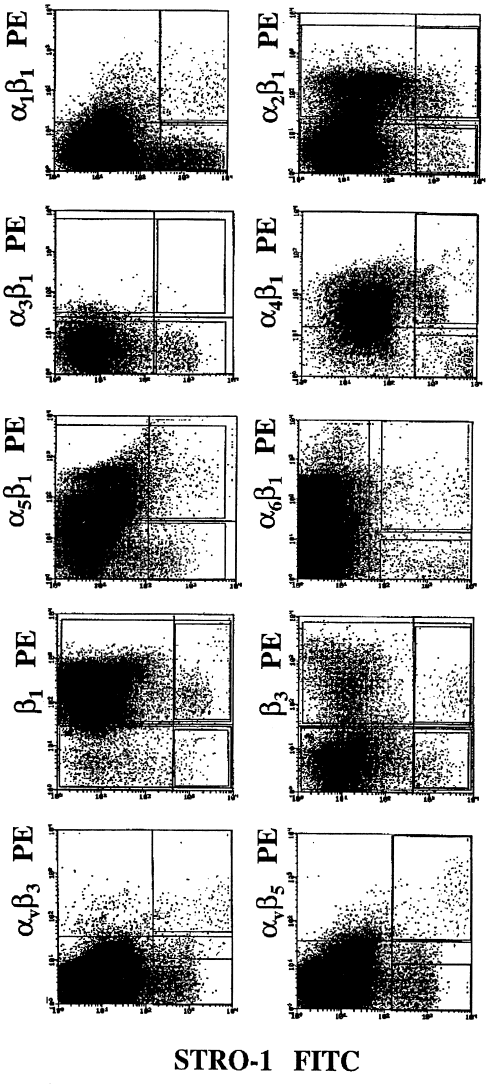

Figure 2. Distribution patterns of the STRO-1 antigen and different integrin cell surface receptors on human BM cells. Dual-color FACS was performed using the monoclonal antibody STRO-1 (FITC) and various antibodies specific to the different integrin molecules (PE), as described in Materials and Methods. Representative frequency histograms of log fluorescence are shown. The vertical and horizontal lines were set to the reactivity levels obtained with the isotype-matched control antibodies 3D3 (PE) and 1A6.12 (FITC), respectively, for each experiment. The STRO-1 population is depicted within the lower right-hand and upper right-hand quadrants representing the STRO-1^+/integrin$^-$ and STRO-1^+/integrin$^+$ subsets, respectively. The histograms were generated from a total of 5×10^4 events collected as list-mode data.

represented in the top and bottom right hand quadrants (Figure 2). We have previously found that BM CFU-F reside only in the STRO-1 bright fraction (approximately 1% of the total STRO-1^+ population), whereas STRO-1 dull to intermediate-expressing cells are mostly nucleated red cells and some B-lymphocytes.[23,56] The results demonstrated that the integrin molecules, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, β_1, β_3, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ were expressed by the majority of the CFU-F population derived from normal marrow aspirates (Figure 3). In contrast, integrin $\alpha_4\beta_1$ was only expressed on a proportion of BM CFU-F (50%), while there was no significant cell surface expression observed for integrin $\alpha_3\beta_1$.

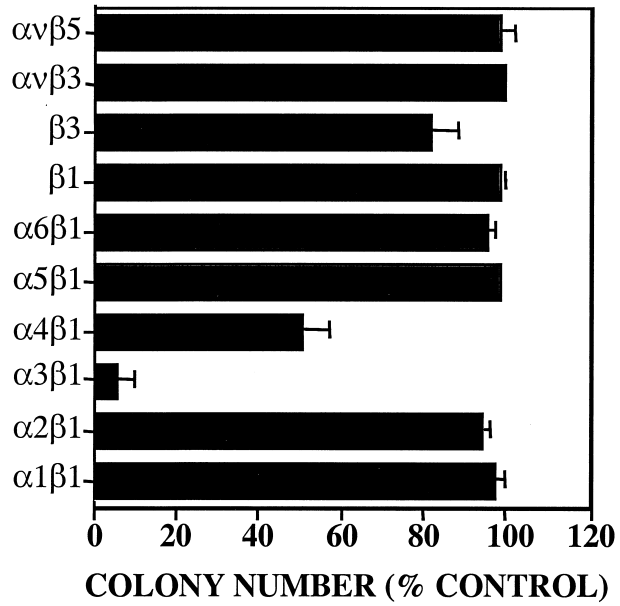

Figure 3. The cell surface expression of integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, β_1, β_3, $\alpha_v\beta_3$, and $\alpha_v\beta_5$) on human BM CFU-F. Human BMMNC were separated into different STRO-1/integrin subfractions by FACS as depicted in the regions shown in Figure 2, where the lower right-hand and upper right-hand quadrants represent the STRO-1^+/integrin$^-$ and STRO-1^+/integrin$^+$ subsets, respectively. Each sorted population was subsequently cultured in the presence of 20% fetal calf serum as described in Materials and Methods. Colony formation for each STRO-1/integrin subset was expressed as a percentage of the total number of colonies generated from the total STRO-1 population. The data represent the mean values (± SEM) obtained in the STRO-1^+/integrin$^+$ subfractions from three separate experiments.

The Growth Potential of BM Stromal Precursor Cells on Collagen, Laminin, and Fibronectin but Not Vitronectin Is Mediated by β_1 Integrins

The collagen receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_v\beta_3$) expressed by CFU-F were assessed for their role in the interaction of stromal precursor cells to different collagen species. Adhesion assays were performed using STRO-1^+ BM cells preincubated with different combinations of the functional blocking antibodies (HB245, AC11, 61.2C4, and 23C6) specific for $\alpha_1\beta_1$, $\alpha_2\beta_1$, β_1, and $\alpha_v\beta_3$, respectively. The ability of BM CFU-F to generate colonies when seeded onto collagen was significantly ($p \leq 0.05$) reduced when pretreated with different combinations of the β_1 integrin antibodies, anti-β1, $-\alpha_1\beta_1$, and $\alpha_6\beta_1$ (**Figure 4**A). Maximal inhibition was observed using a combination of all three antibodies. There was no effect on the clonogenic growth of CFU-F plated onto collagen after incubation with the $\alpha_v\beta_3$-specific monoclonal antibody 23C6.

In similar experiments, a panel of monoclonal antibodies (HB245, AC11, and GOH3) specific for the laminin receptors ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$, respectively) were used to assess the role of integrins in mediating stromal precursor cell proliferation on laminin. A significant ($p \leq 0.05$) reduction in the number of clonogenic colonies was observed on laminin-coated plates in the presence of the β_1 function blocking antibody (61.2C4) or with the antibody combination of anti-$\alpha_1\beta_1$, $-\alpha_2\beta_1$, and $-\alpha_6\beta_1$ with or without 61.2C4 (Figure 4B). In contrast, the clonogenic growth of CFU-F to laminin was not perturbed in the presence of the $\alpha_v\beta_3$-specific antibody, 23C6.

Blocking studies were also conducted to investigate the role

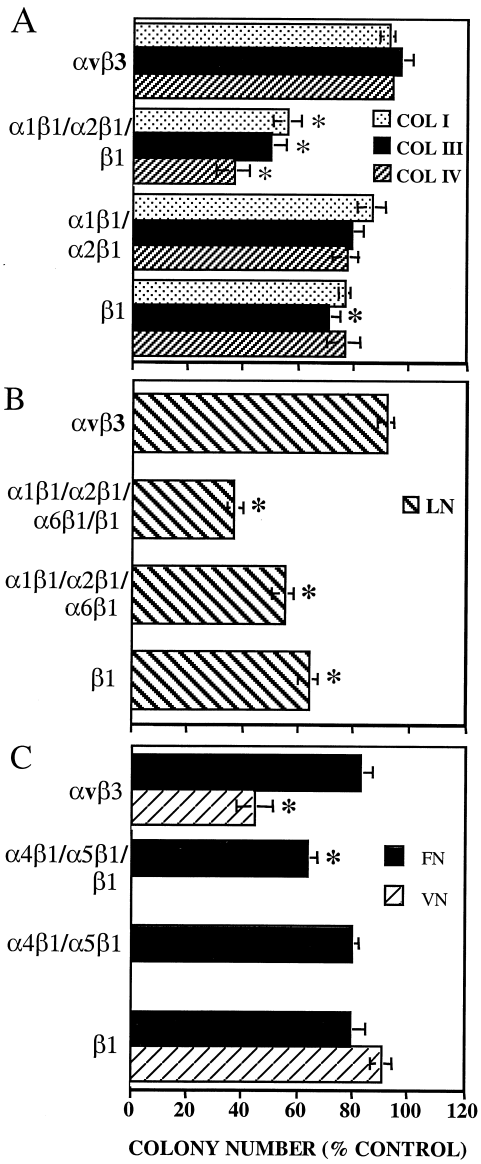

Figure 4. Integrin-mediated adhesion and growth of human BM CFU-F on various matrix glycoproteins. Suspensions of STRO-1-positive BM cells were incubated with combinations of functional-blocking antibodies (HB245, AC11, P4G9, PHM2, GoH3, 61.2C4, and 23C6 specific for $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, β_1, and $\alpha_v\beta_5$, respectively). Clonogenic assays were subsequently performed using plates coated with either: (A) collagen types I, III, IV (COL-I, -III, -IV); (B) laminin (LN); or (C) fibronectin (FN) and vitronectin (VN), as described in Materials and Methods. The results were expressed as a percentage of the total number of colonies formed in the control wells containing the appropriate isotype-matched control antibodies. The level of significance ($*p \leq 0.05$, *t*-test) for colony formation in all conditions was compared with the corresponding control wells.

of different integrin molecules in the growth potential of stromal precursor cells on fibronectin using the monoclonal antibodies $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ (P4G9, PHM2, and 23C6). A significant ($p \leq 0.05$) reduction in colony formation on fibronectin-coated plates was only observed in the presence of a combination of P4G9, PHM2, and 61.2C4 (Figure 4C). As with collagen and laminin, no significant inhibition of colony number was observed for fibronectin when pretreated with anti-$\alpha_v\beta_3$, 23C6. In contrast,

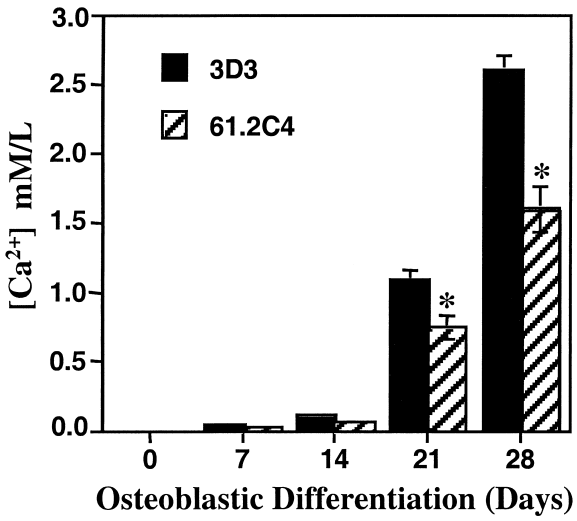

Figure 5. Integrin β1 inhibits the ability of BM stroma to initiate matrix mineralization in vitro. Calcium levels were determined from the adherent stromal cell layers of replicate cultures (n = 4) under osteoinductive conditions (ASC-2P, DEX, and PO_{4i}). Cultures were supplemented with saturating levels of either the monoclonal antibody 61.2C4 or the control isotype-matched antibody (3D3) and then killed at weekly intervals over a period of 28 days. The release of Ca^{2+} from the matrix was measured by treating the cultures under acidic conditions as described in Materials and Methods. The absolute calcium concentration was determined according to a standard curve for calcium and normalized for 10^4 cells per well. Significant differences ($*p \geq 0.05$, *t*-test) in the calcium concentrations for each condition were assessed for at all time points.

a significant decrease ($p \leq 0.05$) in colony number was observed when cells were plated on to vitronectin in the presence of anti-$\alpha_v\beta_3$ but not when incubated with anti-β1.

Osteoblastic Differentiation of Stromal Precursor Cells Is Dependent on β_1 Integrin Function

Primary cultures of BMSC derived from STRO-1-positive sorted CFU-F were placed under osteoinductive conditions to initiate the development of a mineralized matrix in the presence of saturating levels of the β1 blocking antibody (61.2C4) or the isotype-matched control antibody (3D3). Replicate cultures were prepared to monitor the effect of anti-β_1 on the level of Ca^{2+} accumulated in the matrix by the BM stromal cells over a period of 4 weeks. Differences in the Ca^{2+} accumulation between the anti-β_1-treated and the control cultures were evident by the second week of culture. Treated cultures demonstrated a significant ($p \leq 0.05$) decrease in the amount of Ca^{2+} accumulated in comparison with the corresponding control cultures at days 21 and 28 (**Figure 5**).

Discussion

Cellular interactions with the extracellular matrix are thought to orchestrate tissue organization by regulating cell differentiation and function during fetal development and throughout normal adult life.[12,13,18,64] To begin to understand the possible mechanisms that influence BM stromal cell commitment, we attempted to characterize, for the first time, the interactions between different ECM proteins and purified human stromal precursor cells isolated directly from fresh aspirates of normal marrow. In the present study, BM stromal precursor cells were found to possess a high binding affinity and growth potential to the extracellular

matrix components commonly found in the reticular marrow (Col I, III, FN), bone matrix (COL I), endothelial basal laminae (COL IV and LN), and in serum (FN, VN). The stromal precursor cells demonstrated a higher cloning efficiency when seeded onto plates precoated with collagen type IV, fibronectin, vitronectin, and laminin in comparison with collagen types I- and III-coated plates. The differential growth patterns observed on different collagen species may reflect the primitive state of the stromal precursor cells. This is in accord with previous studies, where cultured human trabecular osteoblast-like cells demonstrated a higher binding affinity to fibronectin with respect to their adherence to laminin and collagen type IV.[26,53] Other studies have reported that osteoprogenitors, with the capacity to form mineralized bone nodules in vitro, also showed preferential binding to laminin, whereas rat calvaria osteoblastic-like cells showed a reduced capacity to attach to laminin.[50,51] Differential adhesion and growth to matrix proteins between primitive and mature stromal cell types may be a function of their locality in the bone marrow tissue. Unlike mature osteoblasts that line the bone surfaces, the exact location of BM stromal precursor cells in the marrow spaces is still a matter of conjecture. Histological evidence suggests that multipotential stromal progenitors may be associated with the basal laminae of the marrow sinusoid network.[4] Therefore, the attachment of cells to various matrix glycoproteins may be due to their unique integrin expression patterns depending on their original location, and stage of cellular differentiation, which in turn could influence cell commitment, maturation, and function.

In most respects, stromal precursor cells isolated directly from BM aspirates share a similar integrin expression pattern with various cultured BM stromal cell and bone cell populations.[10,11,26,29,33,53,55,59] Two important exceptions are that stromal progenitors constitutively express the laminin receptor, $\alpha6\beta1$, but lack expression of the collagen/laminin receptor, $\alpha3\beta1$. This is in direct contrast to osteoblast-like cells that express $\alpha3\beta1$ but lack expression of $\alpha6\beta1$. It remains to be seen whether $\alpha3\beta1$ demonstrates a greater affinity for fibrillar collagen types I and III in comparison with the structurally different basement membrane constituent, collagen type IV, which could explain the differential growth pattern observed. This may represent one mechanism by which primitive BM stromal precursor cells could associate with the bone marrow vasculature until environmental cues promote their proliferation and subsequent maturation. During differentiation, the binding affinity of stromal precursor cells to laminin may be altered through the downregulation of the $\alpha6\beta1$ receptor, and may also involve the initial absence of the integrin $\alpha3\beta1$. Subpopulations of stromal precursor cells expressing the fibronectin receptor $\alpha4\beta1$ may also represent transitional progenitor populations undergoing osteogenic differentiation. Immunohistological studies of bone sections have previously shown that a subpopulation of differentiated bone cells express high levels of $\alpha_4\beta_1$ in vivo.[29] The employment of two- and three-color FACS analysis, to subdivide the BM stromal precursor compartment, may reveal subpopulations of stromal progenitors with different maturity and/or developmental potentials.

Integrin interactions are known to be important in the regulation of angiogenesis, haematopoiesis, bone deposition, and remodeling.[12,13,15,18,27,64] We therefore examined the role of individual integrin molecules in stromal cell function and development. Many integrins react with the unique tripeptide (arginine-glycine-aspartate) RGD sequence found in fibronectin, vitronectin, and collagen.[15,27,29,52,54] Synthetic peptides that recognize the RGD sequence are known to inhibit the binding of osteogenic cells to collagen type I and fibronectin in vitro.[1,15,27,29,48] One study demonstrated that synthetic RGD-specific peptides could completely inhibit the binding of cultured human osteoblasts to vitronectin, but only caused a partial inhibition to the adherence of bone cells to fibronectin and collagen.[29] Similar studies have reported that the tetrapeptide RGDS inhibited both bone formation and resorption in cultures derived from fetal rat parietal bones.[27] More recently, Damsky and colleagues have implicated a role for the integrin molecules, $\alpha5\beta1$ and $\alpha3\beta1$, in bone formation, but not the RGD binding integrins $\alpha v\beta3$ and $\alpha v\beta5$, known to mediate bone resorption by osteoclasts.[42] Other $\beta1$ integrin subfamily members, such as $\alpha2\beta1$, have also been reported to be important in early bone matrix formation during osteogenesis, and in chondrocyte survival and differentiation.[54–57] In the present study, we have shown that the adhesion and subsequent growth of stromal precursor cells seeded onto collagen, laminin, and fibronectin are $\beta1$ regulated, while their attachment to vitronectin is mediated by $\alpha v\beta3$ rather than through $\beta1$ interactions. In addition, the ability of human BM stromal cells to initiate matrix mineralization was significantly diminished in the presence of a functional blocking monoclonal antibody to the $\beta1$ integrin subunit, demonstrating a possible role for this integrin subfamily during bone formation. Collectively, these studies suggest that the resorbtion of bone by osteoclasts may be mediated through integrins such as $\alpha v\beta3$ and perhaps $\alpha v\beta5$, while osteoblast development and bone formation may be more dependent on $\beta1$ integrin interactions.

Continued studies into the functions of integrins and other superfamilies of cell adhesion molecules may help identify the precise mechanisms for the control and regulation of stromal cell differentiation. Several groups have demonstrated the efficacy of using BM stromal cultures to generate a calcified bone matrix in vivo.[3,28,30,39] Understanding stromal precursor cell-matrix interactions may eventually lead to the development of appropriate strategies for the successful engraftment of these cells. One possible clinical application of such studies is the use of ex vivo expanded stromal cells to seed biomaterials for use in bone replacement therapies. Evidence exists that the type of biomaterial used or the coating of biomaterials with different ECM proteins has significant effects on the ability of the transplanted BM stromal cells to differentiate into functional bone cells in vivo.[62–64] Furthermore, studies by Sinha and Tuan[58] have shown that bone cells exhibit a differential integrin expression pattern when seeded onto different orthopaedic alloys. Therefore, the coating of implant materials with appropriate combinations of purified ECM proteins or their synthetic mimetics may enhance the adhesion, proliferation, and differentiation of stromal cells in order to facilitate their use in the regeneration and repair of osteogenic defects in vivo.

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